Probing biological interfaces by tracing proton passage across them†

Armen Y. Mulkidjanian*[a,b] and Dmitry A. Cherepanov[c]

Received 21st November 2005, Accepted 7th April 2006
First published as an Advance Article on the web 28th April 2006
DOI: 10.1039/b516443e

The properties of water at the surface, especially at an electrically charged one, differ essentially from those in the bulk phase. Here we survey the traits of surface water as inferred from proton pulse experiments with membrane enzymes. In such experiments, protons that are ejected (or captured) by light-triggered enzymes are traced on their way between the membrane surface and the bulk aqueous phase. In several laboratories it has been shown that proton exchange between the membrane surface and the bulk aqueous phase takes as much as about 1 ms, but could be accelerated by added mobile pH-buffers. Since the accelerating capacity of the latter decreased with increase in their electric charge, it was suggested that the membrane surface is separated from the bulk aqueous phase by a barrier of electrostatic nature. In terms of ordinary electrostatics, the barrier could be ascribed to dielectric saturation of water at a charged surface. In terms of nonlocal electrostatics, the barrier could result from the dielectric overscreening in the surface water layers. It is discussed how the interfacial potential barrier can affect the reactions at interface, especially those coupled with biological energy conversion and membrane transport.

1. Introduction

Das tote, seiner molekularischen Selbstbewegung beraubte Wasser, das sich bei einer Temperatur über 0 °R··· eingepresst befindet, ist ein Äquivalent des in der Kälte zu Eis erstarrten, nur mit dem Unterschiede, dass ersteres (wahrscheinlich weil es noch so viel Molekularbeweglichkeit besitzt, daß sich die Elementarteile desselben polarisch ordnen können, ohne dass ein Austausch stattfindet) seine elektrische Leitkraft beibehalten hat, welche dem Eis mangelt.

Theodor von Grotthuß, 1820.‡

Two hundred years ago, 20-year-old Theodor von Grotthuß has suggested a mechanism of charge transfer along chains of water molecules.[1] It is less known that he persisted with experimental studies of proton transfer in the years to follow.[2,3] In 1820, at the very end of his life, von Grotthuß published a short paper on properties of thin confined water layers.[4] As documented by the epigraph to this section, Grotthuß has realized that the molecules of confined water are more sluggish than those in the bulk phase, but still mobile enough to mediate proton transfer.[4]

A widespread example of confined water is that at electrically charged surfaces. Such hydrated interfaces are intensively studied because of their practical importance. The experimental approach consists routinely in sending a beam across an interface and in measuring the output signal.[5] This approach is used upon neutron scattering,[6,7] neutron reflectometry,[8] X-ray reflectivity,[9,10] and diverse optical techniques.[5,11–13] The approach, however, is not very powerful in probing the water layers beyond the first one(s). The interfacial water in all its "depth" could be still scrutinized by measuring the osmotic stress,[14–16] electric potential changes,[17] hydration forces[18] and, more recently, by applying the atomic force microscopy (AFM).[19–22]

Biological membranes are negatively charged at neutral pH.[23] Accordingly, on one hand, their surfaces represent charged hydrated interfaces. On the other hand, it is possible to follow proton transfer across such interfaces. The reactions of proton ejection and proton binding were experimentally addressed in many membrane enzymes, and, in particular, in bacteriorhodopsin (BR),[24–31] bacterial photosynthetic reaction centers (RCs),[32–37] photosystem II,[38–42] the cytochrome bc_1 complex,[43–46] diverse oxidases,[47–54] nitric oxide reductase,[55] ATP synthase.[56–59] In many experimental set-ups, proton displacements were monitored not only inside the membrane enzymes, but also across the membrane/water interface. As discussed in more detail elsewhere,[60,61] the original aim of the research was to scrutinize the biological energy conversion. At the same time, these studies have provided unique information about the surface water layers, as probed by passing protons. In different labs it has been repeatedly shown that proton transfer between the membrane surface and the bulk aqueous phase proceeds rather slow, at milliseconds,[17,24,27–29,34,62–70] as reviewed elsewhere.[30,60,61,71,72] The retardation was due to the interfacial barrier of electrostatic nature, some properties of which could be elucidated.[60,72–75] This new knowledge about the interfacial water layers can be considered as complementary to that acquired by traditional techniques of surface science.[5,15,18]

In this review, we focus on the charge transfer across the membrane/water interface and on the properties of the surface water layers. The lateral proton transfer along the membrane, as

[a]*A.N.Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, 119899, Russia*
[b]*School of Physics, University of Osnabrück, Fachbereich Physik, Universität Osnabrück, D-49069, Osnabrück, Germany. E-mail: AMULKID@UOS.DE; Fax: +49-(541)-9692656; Tel: +49-(541)-9692506*
[c]*A.N. Frumkin Institute of Physical Chemistry and Electrochemistry, Russian Academy of Sciences, Leninskii prosp. 31, 117071, Moscow, Russia*

† This paper was published as part of the special issue on Proton Transfer in Biological Systems.

‡ The stiff water, which is robbed of its molecular motion by being squeezed into a thin layer at temperatures above 0 °R [=0 °C],··resembles water, which has been frozen into ice by cooling. The only difference is that the former retains its electric conductivity, most probably because its molecules retain some residual mobility that enable them to reorder in the presence of electric field, while the ice lacks it. Theodor von Grotthuß, 1820.

well as the interplay between the lateral and transverse proton transfer are considered in another review,[61] the two articles are mutually complementary.

2. Retarded proton transfer across the membrane/water interface

Licht trennt die Bestandteile vieler ponderablen Verbindungen voneinander und zwingt sie neue Verbindungen mit seinen eigenen imponderablen Elementen (+E und −E) einzugehen, gerade wie es die Pole der Voltaschen Batterie, nur in einem höheren Grade, zu tun vermögen.

Theodor von Grotthuß, 1819.§

In some photosynthetic enzymes the light reaction is coupled with fast proton binding or release. Thereby, on one hand, the flash-induced enzyme reactions can be studied by following the intra-protein proton displacements.[76–78] On the other hand, the resulting proton consumption from the bulk aqueous phase (or the proton release into this phase) can be traced spectrophotometrically by using hydrophilic pH indicators.¶,[76,77,79] Studies of proton binding by diverse RCs (see Fig. 1A for the experiment scheme) have shown that proton exchange between membrane proteins and the pH-dyes in the aqueous phase proceeded slower than expected under assumption of unconstrained diffusion of protons in water.[38,77,79–83] These experiments, however, could not discriminate whether protons were impeded (i) on their way from the bulk water to the membrane surface or (ii) during their penetration through the protein. This ambiguity was cleared up by Drachev and co-workers who studied the flash-induced proton transfer by bacteriorhodopsin (BR) sheets[24] (see Fig. 1B for the scheme of proton transfer in BR). Drachev and co-workers have followed not only the spectral changes (i) of BR proper and (ii) of the pH-indicator *p*-nitrophenol in the solution, but also traced, by using capacitive voltammetry, (iii) the movement of a proton from the buried retinal cofactor to the membrane surface. The proton delivery to the surface followed the formation of the M intermediate of the BR photocycle, whereas the protonation of the water-dissolved pH-indicator was distinctly retarded. Hence, protons were hindered on their way between the surface of the BR membrane and the bulk aqueous phase. The ability of added hydrophilic pH-buffers to accelerate the protonation of the pH-dye also indicated that the kinetic barrier passed, quite paradoxically, not through the protein moiety but through the water phase.[24,66]

Heberle, Dencher and their co-authors have used an even more sophisticated approach to study the same reaction of flash-induced proton release by BR. They used two pH-indicators, namely fluorescein, which was covalently bound to the surface, and pyranine that was dissolved in the solution (see Fig. 1B for the experimental setup). Fluorescein was protonated at <0.1 ms,

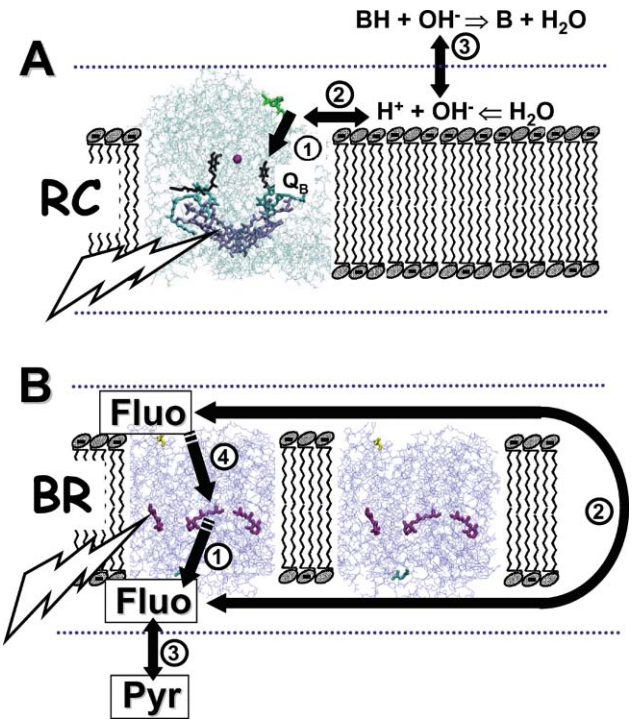

Fig. 1 Comparative presentation of the partial steps of proton binding by the RC of *Rb. sphaeroides* (A, top) and of proton release into the bulk by BR (B, bottom). The numbers indicate the sequence of reaction steps. The dashed lines indicate the interfacial barrier for ions. (A) Proton trapping from the bulk aqueous phase by the RC of *Rb. sphaeroides* (the PDB entry 1AIJ[176] was used on drawing). BH/B, protonated/deprotonated molecules of hydrophilic mobile pH-buffer, respectively. Color code: ubiquinone is shown in black, bacteriochlorophyll in ice-blue, bacteriopheopytin in cyan, the histidine residues that are involved in proton trapping in green. (B) Proton transfer steps in BR (two identical crystal structures of BR trimers,[177] PDB entry 1BRR, are depicted). Fluo, fluorescein, Pyr, pyranine. Colour code: retinal is shown in purple, Lys-129 is marked in cyan, Asp-36 is shown in yellow (see the text for further details). The figure was produced by using the VMD software package.[178] The figure is modified from ref. 60.

concomitant with the formation of the M-state, whereas pyranine was protonated much slower, at ~0.8 ms.[26–28,65] The delayed proton transfer from the surface into the bulk aqueous phase was thereafter confirmed in several other labs.[29,67,70,84]

Proton transfer in the opposite direction, from the bulk water phase into the RCs of phototrophic bacteria *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*, was tracked with native membrane vesicles of these bacteria (chromatophores, see Fig. 1A). Here again the intra-protein proton transfer was complete at ~0.1 ms, whereas the response of diverse pH-indicators in the solution was retarded up to 0.5–1 ms.[34]

The slow rate of proton equilibration between the surface of biological membranes and the bulk water phase has been initially attributed to the damping effect of immobile pH-buffers at the surface, *i.e.* of the ionizable lipid and protein groups.[27,63,71,85–88] Numerous studies with diverse membrane preparations have shown that it was indeed possible to affect the surface proton dynamics by modifying the surface buffer groups.[89–92]

However, if the surface pH-buffers were *alone* responsible for the proton retardation, then the mobile, non-adsorbing pH-buffers or

§ Light splits up many ponderable compounds and forces them to interact with its imponderable (weightless) elements (+*E* and −*E*) in the same way as the poles of a Volta pile, although to a larger extent, are able to do. Theodor von Grotthuß, 1819.

¶ Here it seems appropriate to acknowledge that we owe this experimental set-up to Grotthuß. Besides coining the first low of photochemistry ("Light must be absorbed by a chemical substance in order for a photochemical reaction to take place"),[3] Grotthuß has provided us with the first description of a light-driven separation of electric charges, as documented by the epigraph to this section.

pH-indicators were expected to accelerate proton equilibration with the bulk aqueous phase if added at concentrations of >1–5 μM, *i.e.* when they could kinetically compete with free protons at neutral pH.[34,72,86] As a rule, this was not the case. Only the mono-anionic species accelerated proton equilibration at concentrations of ≥25 μM.[24,70] The di-anions such as phosphate and bromcresol purple were efficient only when added at >100 μM.[34,65,83,93] Pyranine, which carries four negative charges, did not notably accelerate the proton exchange.[65,70,87] The apparent dependence on the electric charge of mobile pH-buffers prompted a suggestion on an interfacial potential barrier of electrostatic origin between the surface and the bulk aqueous phase.[60,72–74] As argued elsewhere[60,74,75] and as discussed in more detail in Section 3 below, the interfacial barrier is just one of emanations of specific properties of surface water and might have a complex physical nature. In terms of ordinary electrostatics, the interfacial barrier could be ascribed to dielectric saturation of the surface water layers at the negatively charged membrane surface.[74] In terms of nonlocal electrostatics, the potential barrier can be described by dielectric overscreening.[75]

Although a rigorous physical description of the barrier is not feasible yet, some quantitative estimates could be inferred from experimental data. In particular, proton transfer across the interfacial barrier is characterized by weak pH-dependence and high activation energy of 30–50 kJ mol^{-1}.[34,65,68,83] As argued elsewhere,[34] both features point to the participation of neutral water in proton transfer across the interface. Indeed, a direct collisional interaction of *mobile* pH-buffers coming from the bulk solution with the *newborn* protons (or proton holes) at the membrane surface should have low activation energy of ≤10 kJ mol^{-1}, as is typical for diffusion-controlled reactions. On the other hand, the observed high activation energy of 30–50 kJ mol^{-1} is characteristic for the protonation/deprotonation of water at neutral pH values. Apparently, the molecules of mobile buffer fail, because of the interfacial potential barrier, to reach the newborn surface protons/proton vacancies before the latter interact with molecules of neutral water that are abundant at the surface.[34] Hence, one gets a two-step mechanism: first the *newborn* charges at the surface interact, in a reaction with high E_a, with neutral water yielding either H_3O^+ or OH^- (depending on the sign of a newborn charge), and only then these charged water species diffuse into the bulk (as depicted in Fig. 1A). The accumulating evidence of facilitated protolysis of neutral water at interfaces (see comments of Beattie[94] and references therein) supports this mechanism.

In a further attempt to reveal the properties of the interfacial barrier, we analysed, by solving a system of diffusion equations and by comparing the solution with the experimental data, which factors determine the rate of the pulsed protonic relaxation at the membrane/water interface of spherical vesicles. The modelling has revealed that an added pH-buffer accelerates the proton equilibration with the bulk phase once its concentration exceeds a certain threshold.[74] The threshold value depends on the barrier height but is independent both of the vesicle size and of the surface buffering capacity. This feature helped to "extract", from the experimental data, the values of the barrier height, as sensed by different penetrating ions. Fig. 2A and 2B show sets of curves, as calculated for different altitudes of potential barrier. The calculated curves are plotted over experimental points reflecting

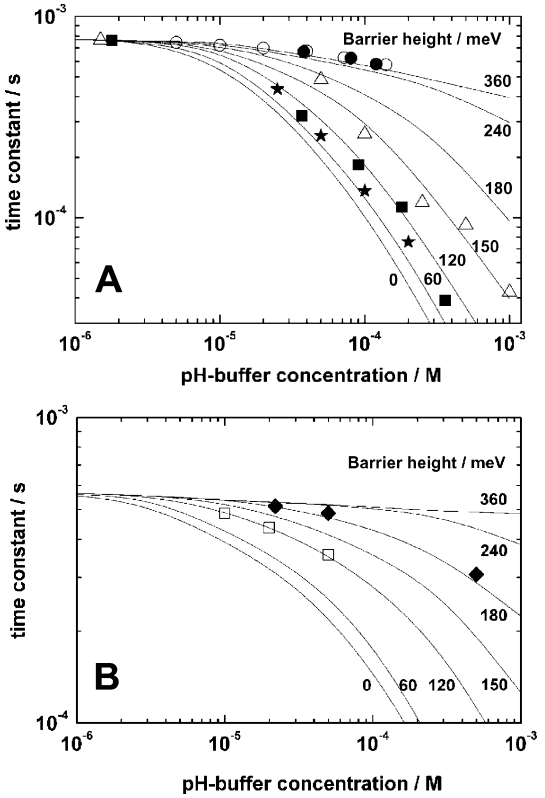

Fig. 2 Interfacial potential barrier as function of the electrical charge of penetrating ion. In both panels, the set of seven curves was calculated for various heights of the potential barrier for mobile buffer (U_C^{max} = 0, 60, 120, 150, 180, 240 and 360 meV correspond to the curves plotted sequentially from the left to the right) at a given value of the potential barrier U_H^{max} for H^+ ions of $U_H^{max} = U_{OH}^{max}$ = 120 meV (see ref. 72 for further details). (A) Dependence of proton relaxation time at the BR membrane on the concentration of added mobile buffer. The figure corresponds to Fig. 4B of ref. 72. The experimentally measured time of pyranine protonation by the BR-ejected protons is shown by circles (solid circles represent the data from ref.70 and open circles correspond to data from ref. 65), the response time of *p*-nitrophenol[24] is shown by stars, the acceleration of proton relaxation (as measured by pyranine) by added MES[70] and phosphate[25] is shown by squares and triangles, respectively. The response time of indicators in the bulk was re-calculated from the experimental kinetics by accounting for the time needed by BR to eject a proton to the surface (see Fig. 1B). (B) Acceleration of proton relaxation in chromatophores from *Rb. sphaeroides* by mobile buffers. The figure corresponds to Fig. 5 of ref. 72. The response time of BCP is shown by circles and the acceleration of the 20 μM BCP response by MES is shown by squares (the experimental data were taken from ref. 34 and corrected for the time of proton transfer from the surface to the Q_B ubiquinone at 100 μs, see Fig. 1A).

the dependence of ion equilibration rate on its concentration, as obtained for different ion species. As it follows from Fig. 2A and 2B, the magnitude of the barrier depends almost linearly on the electric charge of the penetrating ion and varies between 0.09 eV for *p*-nitrophenol and MES (with charge of −1) and more than 0.36 eV for pyranine (with charge of −4). This linear dependence substantiates the predominantly electrostatic nature of the interfacial barrier.

3. Dielectric saturation and dielectric overscreening in the surface water layers

Da die Elementarteilchen einer solchen Flüssigkeit von der zunächst liegenden Elementarteilchen nach allen Seiten polarisch angezogen und abgestoßen werden, so kann vielleicht ein beständiger Austausch der heterogenen Elemente, den man durch einen elementarpolarischen Zirkel (etwa so  *) vorstellen könnte, in derselben stattfinden···*

Theodor von Grotthuß, 1819 (ref. 3).**

Water is a rather unusual liquid. Its physical properties are governed, as it has been first realized by von Grotthuß[1] (see the epigraph to this section), by networks of hydrogen bonds and by strong dipole–dipole interactions.[95–98] In terms of ordinary electrostatics, the interfacial potential barrier could owe to dielectric saturation in the surface water layers.[73,74] Electrochemists have long claimed that the dielectric permittivity (ε) of the first hydrating water layer at a charged surface is on the order of 4–6.[99] The dielectric permittivity at the surface of lipid bilayers has been reported to be on the order of 10–30.[100,101] Teschke and co-workers have quantified the thickness of the low-polarizable surface water layer from the electrostatic immersion of highly polar silicon nitride and cobalt-coated AFM tips at negatively charged mica surface.[20] They have calculated that the ε value changed from 6 at the surface to 80 at the distance of 10 nm. Further evidence of decreased ε at the surface can be inferred from data on the conductivity of acidic monolayers.[102,103] Here the surface conductivity was studied as function of the surface density of anionic groups. The conductivity increased sharply when the edge-to-edge distance between the neighbouring anionic groups approached ~7 Å, both in monolayers of fatty acids[102,103] and of acidic DL-α-phosphatidyl-L-serine dipalmitoyl.[104,105] This effect was not observed when DL-α-phosphotidylcholine, dipalmitoyl, which does not have an acid group, was studied.[105] The increase in surface conductivity correlated with a sharp rise of the surface electrostatic potential.[102,103] The latter results were interpreted within the framework of phenomenological capacitor models where two or three dielectric layers have been considered.[102] Apparently, at high density of surface anionic groups the effective dielectric constant (ε_{eff}) of water at the interface decreased up to 6–7. Oliveira Jr. and co-workers[102,103] have suggested that at distances below the critical one of ~7 Å, the water molecules got the opportunity to link up two neighbouring acid groups, that would led to the "freezing" of the water at interface (see also ref. 106 and references therein). The formation of water-bridged hydrogen-bonded networks is supported by the pulsed field gradient NMR measurements of proton transfer at the surface of BR membranes. Here the maximal proton transfer rate was observed with fully hydrated samples and the translational proton jump distance at the surface was three times larger than that observed in the bulk water.[107] These observations might reflect the formation of low-barrier proton-conducting water-bridges between the charged groups at the surface.[61,108] The "rigidity" of such hydrogen-bonded networks follows not only from the neutron scattering data,[107] but also from the exponential increase in surface potential, reflecting the decrease in the effective dielectric permittivity.[102,103]

The importance of surface charge for water polarization follows from the observations of Ishino and co-workers who have shown that the negatively charged silicon nitride tips were attracted at small separations *both* to the positively and negatively charged Langmuir–Blodgett monolayers (–NH_2 and –COOH functional groups) but not to the neutral stearyl amide (–$CONH_2$) and stearyl alcohol (–OH) monolayers.[109] The observed dependence of the attraction on the surface charge might be caused by ordering of the polarized water at the distances of few nanometers from the charge interface, *both* on the positively and negatively charged surfaces, leading to dielectric saturation. In the case of a neutral surface, the ordering is likely to be restricted to the first hydration layer.

The AFM experiments of Teschke *et al.*[20] were performed at low ionic strength, either in pure water or in the presence of 1 mM of various salts. The ε profiles, as obtained by these authors, could be, however, extrapolated to higher, biologically relevant ionic strengths.[74] As discussed in more detail in the latter work, the decreased ε at a charged surface could lead to a potential barrier of 0.1–0.3 eV for monovalent ions some 1 nm away from the membrane surface. Such a barrier is large enough to notably retard the ion transfer between the membrane surface and the bulk aqueous phase.[74]

The strong intermolecular correlations in water[98,110,111] do set, however, limits on treatment of short-range interactions in water in the framework of ordinary electrostatics. To account for such correlations, a theory of nonlocal dielectric permittivity with a spatial dispersion has been put forward by several authors.[112–116] In this theory, the dielectric displacement $\vec{\boldsymbol{D}}$ at a given point $\boldsymbol{r}$ depends, *via* the nonlocal dielectric function $\varepsilon(\boldsymbol{r},\boldsymbol{r})$, on the electric field in the whole volume occupied by the dielectric medium:

$$\vec{\boldsymbol{D}}(\boldsymbol{r}) = \int_V \varepsilon(\boldsymbol{r},\boldsymbol{r})\boldsymbol{E}\vec{\boldsymbol{D}}(\boldsymbol{r}')d^3\boldsymbol{r}'$$

The nonlocal dielectric function of isotropic water has been obtained by molecular dynamics simulations.[110,117] It was found to be negative at wavelength values between 2 and 12 $Å^{-1}$. The negative sign of dielectric function indicates overscreening.[118,119] The molecular reason for overscreening is the coupling between spatial and orientational correlations in liquid water resulting in an enhancement of the polarizability at certain wavelength.[120] The negative sign of dielectric permittivity makes the behaviour of aqueous electrolyte solutions similar to that of ferromagnetic materials and high-temperature superconductors.[75,118,119]

In other words, water molecules have a tendency to form highly ordered molecular clusters because of intermolecular correlations.[98] This intrinsic property manifests itself in numerous physical, electrochemical and interfacial phenomena. Particularly, the behaviour of water at an interface differs drastically from that of bulk water because of the strong organizing impact of the surface on the water structure. When spread over an electrically charged surface, water tends to form layered structures, which were demonstrated by atomic force microscopy (AFM)[19,22] and by X-ray reflectivity.[9] A distinctive feature of the interfacial water

** As the elementary particles in such a liquid, depending on their polarity, are attracted and repelled by the surrounding elementary particles, one can imagine an interaction between the heterogeneous elements in such a liquid as a circle where the elements are arranged according to their polarity (approximately in the following way: ). Theodor von Grotthuss, 1819 (ref. 3).

confined in thin films is its high fluidity[121–123] that differs sharply from the behaviour of non-associative liquids; the latter have a tendency to be in a solid-like state under similar conditions.[124,125] Water behaves similarly at the surface of biological membranes, in particular BR sheets: by using neutron scattering Dencher and co-workers have shown that the translational diffusion of the surface water molecules was anisotropic and occurred only parallel to the membrane plane.[107,126] Another enigmatic interfacial phenomenon, as first elegantly demonstrated by Israelachvili and Pashley,[127] is the appearance of force oscillations in electrolyte solutions squeezed in nanoscopic films.[128] The origin of oscillations, which depended on salt concentration and which were taken as evidence for the high organization of interfacial water, has remained elusive.[121–123,129–131] The oscillations could not been derived from the classical Derjaguin–Landau–Verwey–Overbeek (DLVO) theory. The latter considers an ideal electrolyte solution in a structureless polar solvent where the interaction of particles includes only monotonic van der Waals attraction at short distances and electrostatic repulsion at large separations.[18] The consistent theoretical description of oscillations and of some other anomalies of interfacial water has been recently accomplished by using nonlocal dielectric function of isotropic water.[75] According to this approach, the redistribution of mobile ions in an electrolyte solution exerts a positive feedback on the overscreening response of water. This feedback can cause resonant oscillations of the electrostatic potential in the vicinity of a charged surface that makes the consideration of nonlinear effects of dielectric saturation and of volume exclusion obligatory. It should be mentioned that these potential oscillations are analogous to the spontaneous appearance of spin density waves in ferromagnetic systems and to the appearance of charge density waves in plasma and in high-temperature superconductors.[118,119]

Fig. 3 shows the oscillations in a squeezed electrolyte for different electrolyte ionic strengths, as calculated elsewhere.[75] These oscillations are in a remarkable agreement with the experimentally measured oscillating forces between negatively charged macroscopic mica cylinders (see the insert to Fig. 3): at ionic strength of $\leq 10^{-4}$ M, the interaction of mica surfaces in KCl solution was repulsive at great separations and monotonically attractive at short distances; at the intermediate concentration of 10^{-3} M, sharp oscillations of repulsive and attractive force were observed in the interfacial layer of 2 nm thickness; at ionic strength of $\geq 10^{-2}$ M, a strong hydration repulsion dominated other forces in the layer of 3–5 nm thickness.[121,127,132]

The surface water layering is likely to affect many interfacial reactions and therefore has to be routinely taken into account. As already mentioned in Section 2, the examination of charge transfer reactions at the interface of biological membranes revealed a linear correlation between the electric charge of a penetrating ion, on one hand, and the height of the effective interfacial barrier as sensed by this ion, on the other hand.[72] Exactly this kind of dependence is expected if the kinetic barrier is due to the potential barrier in the surface water layers, as calculated by using the nonlocal approach.[75]

It is worth mentioning that the described interfacial potential barrier is only one of many emanations of the peculiar properties of surface water. The exact relation of the barrier to the "solute free" surface water,[15] and to other surface phenomena[133–135] has to be established yet.

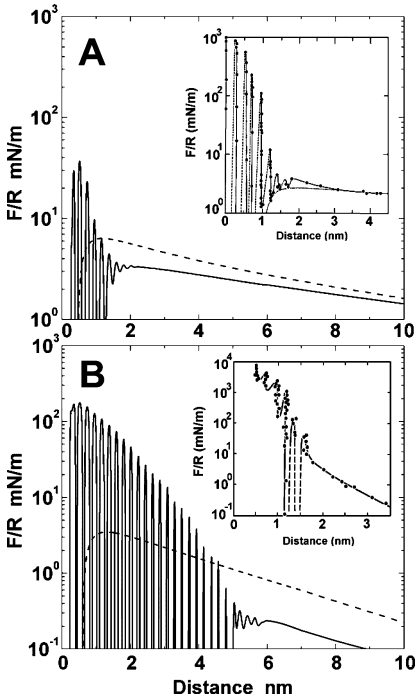

Fig. 3 The normalized force between two charged cylinders in an electrolyte solution as function of the separation distance. The figure corresponds to Fig. 2 of ref. 75. The interaction force F of a pair of crossed cylinders of radius R, which is large compared to the distance of their minimal approach L, was calculated by the interaction free energy Ω of two parallel charged walls using the Derjaguin approximation $F(L) = 2\pi R\Omega(L)$. The free energy Ω was calculated as described in ref. 75. The solid lines show the interaction force for the nonlocal dielectric function, as described in ref. 75, the dotted lines were obtained with the static dielectric permittivity $\varepsilon = 78$. The surface charge density σ was 0.016 C m^{-2}, the electrolyte solubility $c_{max} = 5$ M, and the empirical parameter $a = 30$. Curves in (A) and (B) correspond to the ionic strength of 10^{-3} and 10^{-2} M, respectively. Insertions to (A) and (B) show the experimental force–distance curves measured in 1 mM and 1 M KCl solutions (data taken from ref. 127 and ref. 132, respectively).

4. Some implications and outlook

Der Sauerstoff eines jeden Wasseratomes wird nämlich selbst negativ,· · · und der Wasserstoff eines jeden Wasseratomes wird selbst positiv elektrisch. Es entsteht nämlich eine molekularpolarische Reihe +–+–+–+–, und durch den elektrischen Zustand der Atome selbst wird ein wechselseitiger Austausch der Elementarteile aller Atome bewirkt.

Theodor von Grotthuß, 1820 (ref. 4).††

4.1 Biological energy conversion

The energy transduction in biological membranes is based on the ability of certain redox- and light-driven enzymes to pump protons across the membrane. In the simplest case of bacterial cells, protons are pumped out, into the surrounding medium. As a

†† The oxygen of each water molecule is electrically negative, while the hydrogen is positive. This leads to the formation of a molecular-polar array +–+–+–+–, so that the mutual interaction between the elementary parts of all molecules is affected by their electrical state. Theodor von Grotthuss, 1820 (ref. 4).

result of this charge separation, one side of the membrane becomes *n*egatively charged, whereas the other side charges *p*ositively, so that one can speak about *n*- and *p*-sides of an energy-transducing membrane. The transmembrane difference in the electrochemical potential of hydrogen ions ($\Delta\tilde{\mu}_{H^+}$) drives the energy-consuming enzymes, the ATP synthase in the first place.[136–138] It has been widely discussed that $\Delta\tilde{\mu}_{H^+}$, as experimentally estimated from the measured $\Delta\psi$ and "bulk" pH values was insufficient to drive ATP synthesis on many occasions,[138–142] and especially in the case of alkaliphilic bacteria.[143] The existence of the interfacial potential barrier helps to solve this fundamental bioenergetic conundrum. The height of the interfacial barrier for protons was found to be about 0.12 eV.[72] Because of the barrier, the surface proton activity, as sensed by membrane enzymes, might deviate from that measured in the adjoining water phase. In particular, the proton concentration at the *p*-surface of energy-transducing membranes should increase at steady state.[60,61,74] Thus, *in vivo* the driving force beyond ATP synthesis is the *surface-to-surface* $\Delta\tilde{\mu}_{H^+}$ that can be defined as

$$\Delta\tilde{\mu}_{H^+}{}^S = F\Delta\psi - 2.3RT\Delta pH^S$$

In general, the *surface-to-surface* $\Delta\tilde{\mu}_{H^+}{}^S$ is expected to be larger than the *bulk-to-bulk* $\Delta\tilde{\mu}_{H^+}$. In more detail the implications for bioenergetics are considered elsewhere.[60,61,74]

4.2 Ion transfer by gramicidin

Another relevant example is the ion transfer through the gramicidin A channel. The proton conductance along this channel, as going along a file of water molecules (see the epigraph to this section for a description of such a file by von Grotthuß), was shown to be limited by the events at the membrane/water interface.[144,145] In particular, the conductance has been shown to depend on proton activity as $[H^+]^{0.75}$ over a range of five pH units.[145] A plausible explanation is that the proton flux to the channel mouth has a considerable surface contribution; in such case the theoretical analysis predicts a flatter dependence of the conductance on proton concentration, as compared to the case of isotropic diffusion in the three-dimensional semi-space.[71]

As considered in ref. 72 and as discussed above in Section 2, the interfacial potential barrier is sensed by any ion. This might explain why the conductance of the already mentioned gramicidin A for K^+ ions, at the applied voltage of $\geq$200 mV, was limited by the ion diffusion in the external water phase.[146] Quantitative analysis of the data, based on using homogeneous diffusion coefficients for permeate ions, yielded a capture radius for the channel mouth of only about 0.02 nm,[146] an order of magnitude smaller than it is seen in the crystal structure. A probable reason of this controversy might be the anisotropic character of the ion diffusion coefficient (tensor), with its normal component being 10^3 fold smaller that the lateral one.

4.3. Atomic force microscopy

Another case where the polarization of the interfacial water seems to manifest itself is the electrostatic interaction of AFM tips with charged surfaces. The radius of standard SiN tips used in the atomic force microscopy is 2–5 nm, as determined by the transmission electron microscopy and by the AFM itself.[147,148] This estimate is in good correspondence with the resolution of AFM. However, the *calculations* of the effective radius from the extent of electrostatic repulsion at the interface yielded an estimate of 100–300 nm for the radii of similar tips.[149] This apparent contradiction can be solved by ascribing a lower effective ε (ε_{eff}) value to the interfacial water layer. As noted above, the layering of the surface water was directly observed by AFM when carbon nanotube probe was combined with highly sensitive dynamic measurement scheme.[19,22]

Generally, the usage of ε_{eff} of 78, as in homogeneous water, is improper when considering events at interface. Such a high ε_{eff} value can lead to underestimation of electrostatic forces. Because of the image charge, ε_{eff} can hardly be higher than 40 at the interface between water and a non-polar medium.[150,151] Any microscopic pre-organisation of water at interface, especially pronounced if the membrane surface is charged, is expected to decrease ε_{eff} further. One can argue that the application of the classical Poisson–Boltzmann equation with fixed ε_{eff} of 78 has yielded acceptable results upon modelling of surface reactions in many cases. Such modelling, however, implies routinely several unrestricted parameters. In the simplest case of a lipid membrane, these are surface charge density, surface electrostatic potential, position and thickness of the membrane, dielectric constant of the membrane, effective radii of chargeable groups and their specific affinity to protons. Because these parameters are roughly independent of each other, one has a considerable degree of freedom in the fitting of experimental data. The examples, which we have chosen above and which provide evidence of a lower dielectric permittivity at the surface, represent either very well studied systems (gramicidin A) or systems, where at least some fit parameters can be independently estimated (*e.g.* the sizes of the gramicidin A mouth and of the AFM tip).

In the practice, it is difficult to account for nonlocal electrostatic effects, as described above in Section 3, upon numerical modelling of complex interfacial phenomena, such as ligand binding or peptide adsorption. Still it is advisable to take $\varepsilon_{eff} < 40$ for the surface water layer upon such calculations. In this case, the values of other fit parameters would be closer to the true ones than when ε_{eff} of 78 is used. The same phenomenological approach is widely used in protein electrostatics, where ε_{eff} values in the range of 10–20, which can hardly be rigorously justified, give the best correspondence with experimental data upon simple calculations.[152–155]

4.4 Calcium pumps

As noted above, the potential barrier depends linearly on the charge of penetrating ion and is therefore higher for ions carrying several charges. In the case of calcium pumps, the barrier might then account for the locally elevated concentration of Ca^{2+} ions at the membrane surface close to the pump outlets.[156,157] Upon studying Ca^{2+} efflux in rat cardiac myocytes, Meethal and co-workers have simultaneously recorded (i) the Ca^{2+} current and (ii) the Ca^{2+} transients as measured by a fluorescent dye fura 2.[158] The transients delayed by 1.3 ms beyond the current. The delay was explained by involvement of unidentified Ca^{2+}-buffering groups at the surface. Although such groups might be involved, the similarity with the proton behaviour at the membrane surface, as described in Section 2, prompts a suggestion that Ca^{2+} ions could be retarded on their way into the bulk aqueous phase by the interfacial potential barrier.

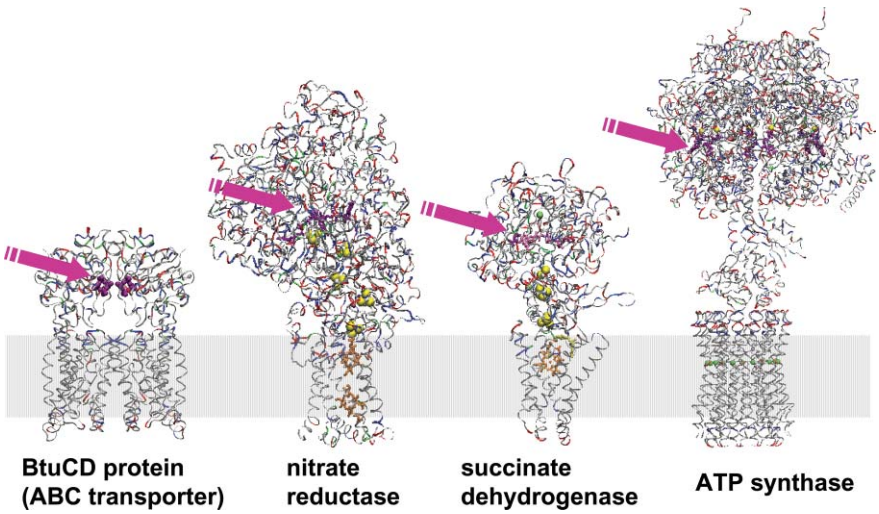

Fig. 4 Binding sites of non-transportable polyanions in several membrane enzymes with resolved crystal structures (the enzymes of *E. coli* were chosen where possible). The 3D structures of the *E. coli* BtuCD protein, an ABC transporter mediating vitamin B12 uptake (PDB entry 1L7V[173]), the *E. coli* succinate dehydrogenase (PDB entry 1NEK[164]), the *E. coli* nitrate reductase (PDB entry 1Q16)[165] and a composite model of a F_OF_1-type ATP synthase (the X-ray structure of the F_1 part of the bovine H^+-ATP synthase (PDB entry 1H8E[179]) is combined with the crystal structure of the F_O part of the *Ilyobacter tartaricus* Na^+-ATP synthase (PDB entry 1YCE[180]) are depicted. Colour code: acidic residues (Asp and Glu) are marked in red, basic residues (Arg and Lys) are shown in blue, and histidine residues are marked in green to reveal the hydrophobic membrane segments. The binding sites of anionic substrates are highlighted in purple and indicated by purple arrows. Purple colour is used for the cyclotetravanadate molecules at the ATP-binding sites of BtuCD protein, for the molybdopterin–guanine dinucleotides and molybdenum in the nitrate reductase, for oxaloacetate in the substrate-binding site of the succinate reductase, and for adenine nucleotides in the F_OF_1-ATP synthase. The iron–sulfur clusters are shown in yellow, hemes in orange, magnesium (in the F_1 part of the F_OF_1-ATP synthase) in yellow, sodium (in the F_O part of the F_OF_1-ATP synthase) in green, quinone (in the succinate hydrogenase) in yellow. The figure was produced by using the VMD software package.[178]

4.5 Polyanions at the membrane interfaces

The impact of the interfacial electrostatic barrier is especially pronounced for polyanionic species (*i.e.* ATP, ADP, phosphate anions, carbonic acids *etc.*). When approaching the membrane surface, these anions, besides crossing the interfacial barrier, have to overcome the repulsion by the negatively charged membrane. It is not surprising that the transport of nucleotides by the mitochondrial ADP/ATP carrier, the best studied anion transporter, is facilitated (1) by direct delivery of nucleotides to the carrier through an enzyme–enzyme exchange[159,160] and (2) by the positive charge of the nucleotide-binding cavities.[161]

Of special interest are those ions, which, on one hand, serve as catalytic substrates of membrane enzymes but, on the other hand, are not carried across the membrane. In the available crystal structures, the binding/catalytic sites for such ions are located far away from the membrane/water interface (see Fig. 4). In the case of redox enzymes, electron transfer chains, as formed by numerous redox centers, connect the peripheral substrate-binding sites with the membrane-embedded machinery of electron/proton coupling. Such arrangement is found in the fumarate reductase,[162,163] succinate dehydrogenase,[164] nitrate reductase,[165] and many other enzymes, as surveyed elsewhere.[166] In the case of F-type ATP synthase,[167–170] P-type ATPases,[171] transhydrogenase,[172] and ABC transporters[173] mechanical gears are used to connect the catalytic sites with the membrane. This design might have several reasons. One is the domain structure of membrane enzymes with recruitment of whole globular modules in the course of evolution. Another reason might be the crowding at the membrane surface.[160,174,175] Besides, however, the remote position of the catalytic centers might be due to electrostatic constrains. It is imaginable that the kinetic losses of bringing polyanions into the "hostile" polarized interfacial water layer are larger than the evolutionary "investments" needed to construct the transmission gadgets. If so, the position of these substrate-binding centers, >2 nm away from the membrane/water interface, might mark the boundary from which the "bulk" aqueous phase stretches out, *at least from the enzymes' point of view.*

Acknowledgements

The authors are indebted to Cristiano Viappiani, Thomas Gensch, and Joachim Heberle for the opportunity to contribute to this special issue of *Photochemical and Photobiological Sciences* devoted to the 200th anniversary of the von Grotthuß breakthrough. Baron Christian Johann Dietrich Freiherr von Grotthuß, known to the scientific world under the chosen name Theodor, was a Baltic nobleman and a subject of Russia. His ancestors moved to Lithuania from Münster (Westphalia). These biographical details are meaningful for the authors who have spent last several years by touring between Moscow, Russia, and Osnabrück in German Münsterland.

The authors are thankful to Drs P. Ädelroth, Y. N. Antonenko, E. Bamberg, A. V. Bogachev, P. Brzezinski, N. A. Dencher, L. A. Drachev, O. Gopta, M. Gutman, J. Heberle, W.Junge, D. Kell, L. I. Krishtalik, T. Krulwich, H. Michel, D. Oesterhelt, P. Pohl, V. Skulachev, H.-J. Steinhoff, A. Warshel, R. J. P. Williams, C. A. Wraight and L. S. Yaguzhinsky for useful discussions

and critical comments. Numerous stimulating talks with the late Dr Andrey Kaulen are especially appreciated.

A.M. is thankful for support to the Volkswagen Foundation, INTAS (2001-736), and the Deutsche Forschungsgemeinschaft (Mu-1285/1, SFB 431-P15, 436-RUS-113/210, Mercator professorship). D.C. was supported by INTAS (2001-736), the Alexander von Humboldt Foundation and the Deutsche Forschungsgemeinschaft.

References

1 T. von Grotthuß, Die Zersetzung des Wassers und der in ihm gelösten Körper durch galvanische Elektrizität, in *Abhandlungen über Elektrizität und Licht von Theodor von Grotthuß*, ed. R. Lüther and A. von Oettingen, Vorlag von Wilhelm Engelmann, Leipzig, 1906, pp. 3–12.
2 T. von Grotthuß, Chemische-galvanische Beobachtungen, in *Abhandlungen über Elektrizität und Licht von Theodor von Grotthuß*, ed. R. Lüther and A. von Oettingen, Vorlag von Wilhelm Engelmann, Leipzig, 1906, pp. 13–18.
3 T. von Grotthuß, Über die chemische Wirksamkeit des Lichtes und der Elektrizität, in *Abhandlungen über Elektrizität und Licht von Theodor von Grotthuß*, ed. R. Lüther and A. von Oettingen, Vorlag von Wilhelm Engelmann, Leipzig, 1906, pp. 94–181.
4 T. von Grotthuß, Merkwürdige Zersetzung des Wassers durch Wasser im Kreise der Voltaischen Säule, in *Abhandlungen über Elektrizität und Licht von Theodor von Grotthuß*, ed. R. Lüther and A. von Oettingen, Vorlag von Wilhelm Engelmann, Leipzig, 1906, pp. 182–189.
5 M. A. Leich and G. L. Richmond, Spiers Memorial Lecture. Recent experimental advances in studies of liquid/liquid interfaces, *Faraday Discuss.*, 2005, **129**, 1–21 (see also other contributions in the same issue of *Faraday Discuss.*, the whole issue is devoted to dynamics and structure of the liquid–liquid interface).
6 G. Zaccai, The effect of water on protein dynamics, *Philos. Trans. R. Soc. London, Ser. B*, 2004, **359**, 1269–75.
7 M. Weik, U. Lehnert and G. Zaccai, Liquid-like water confined in stacks of biological membranes at 200 K and its relation to protein dynamics, *Biophys. J.*, 2005, **89**, 3639–46.
8 A. Zarbakhsh, A. Querol, J. Bowers and J. R. Webster, Structural studies of amphiphiles adsorbed at liquid–liquid interfaces using neutron reflectometry, *Faraday Discuss.*, 2005, **129**, 155–67.
9 L. Cheng, P. Fenter, K. L. Nagy, M. L. Schlegel and N. C. Sturchio, Molecular-scale density oscillations in water adjacent to a mica surface, *Phys. Rev. Lett.*, 2001, 87:156103.
10 G. Luo, S. Malkova, S. V. Pingali, D. G. Schultz, B. Lin, M. Meron, T. J. Graber, J. Gebhardt, P. Vanysek and M. L. Schlossman, X-Ray studies of the interface between two polar liquids: neat and with electrolytes, *Faraday Discuss.*, 2005, **129**, 23–34.
11 C. L. Beildeck, W. H. Steel and R. A. Walker, Surface charge effects on solvation across liquid/liquid and model liquid/liquid interfaces, *Faraday Discuss.*, 2005, **129**, 69–80.
12 J. Liu, X. Shang, R. Pompano and K. B. Eisenthal, Antibiotic assisted molecular ion transport across a membrane in real time, *Faraday Discuss.*, 2005, **129**, 291–99.
13 Z. Samec, A. Trojanek and P. Krtil, Dynamics of phospholipid monolayers on polarised liquid–liquid interfaces, *Faraday Discuss.*, 2005, **129**, 301–13.
14 V. A. Parsegian, R. P. Rand, N. L. Fuller and D. C. Rau, Osmotic stress for the direct measurement of intermolecular forces, *Methods Enzymol.*, 1986, **127**, 400–16.
15 V. A. Parsegian, Protein-water interactions, *Int. Rev. Cytol.*, 2002, **215**, 1–31.
16 H. I. Petrache, I. Kimchi, D. Harries and V. A. Parsegian, Measured depletion of ions at the biomembrane interface, *J. Am. Chem. Soc.*, 2005, **127**, 11546–47.
17 Y. N. Antonenko, O. N. Kovbasnjuk and L. S. Yaguzhinsky, Evidence in favor of the existence of a kinetic barrier for proton transfer from a surface of bilayer phospholipid membrane to bulk water, *Biochim. Biophys. Acta*, 1993, **1150**, 45–50.
18 J. N. Israelachvili, *Intermolecular and Surface Forces*, Academic Press, London, 1992.
19 S. P. Jarvis, T. Uchihashi, T. Ishida, H. Tokumoto and Y. Nakayama, Local solvation shell measurement in water using a carbon nanotube probe, *J. Phys. Chem. B*, 2000, **104**, 6091–94.
20 O. Teschke, G. Ceotto and E. F. deSouza, Interfacial water dielectric-permittivity-profile measurements using atomic force microscopy, *Phys. Rev. E: Stat. Phys., Plasmas, Fluids, Relat. Interdiscip. Top.*, 2001, **6401**, art. no. 011605-05-10.
21 O. Teschke, G. Ceotto and E. F. de Souza, Rupture force of adsorbed self-assembled surfactant layers-Effect of the dielectric exchange force, *Chem. Phys. Lett.*, 2001, **344**, 429–33.
22 T. Uchihashi, M. Higgins, Y. Nakayama, J. E. Sader and S. P. Jarvis, Quantitative measurement of solvation shells using frequency modulated atomic force microscopy, *Nanotechnology*, 2005, **3**, S49–S53.
23 J. F. Nagle and S. Tristram-Nagle, Structure of lipid bilayers, *Biochim. Biophys. Acta*, 2000, **1469**, 159–95.
24 A. L. Drachev, A. D. Kaulen and V. I. Skulachev, Correlation of photochemical cycle, H^+ release and uptake, and electric events in bacteriorhodopsin, *FEBS Lett.*, 1984, **178**, 331–36.
25 S. Grzesiek and N. A. Dencher, Time-course and stoichiometry of light-induced proton release and uptake during the photocycle of bacteriorhodopsin, *FEBS Lett.*, 1986, **208**, 337–42.
26 J. Heberle and N. A. Dencher, Bacteriorhodopsin in ice. Accelerated proton transfer from the purple membrane surface, *FEBS Lett.*, 1990, **277**, 277–80.
27 J. Heberle and N. A. Dencher, Surface-bound optical probes monitor protein translocation and surface potential changes during the bacteriorhodopsin photocycle, *Proc. Natl. Acad. Sci. U. S. A.*, 1992, **89**, 5996–6000.
28 J. Heberle, J. Riesle, G. Thiedemann, D. Oesterhelt and N. A. Dencher, Proton migration along the membrane surface and retarded surface to bulk transfer, *Nature*, 1994, **370**, 379–82.
29 U. Alexiev, R. Mollaaghababa, P. Scherrer, H. G. Khorana and M. P. Heyn, Rapid long-range proton diffusion along the surface of the purple membrane and delayed proton transfer into the bulk, *Proc. Natl. Acad. Sci. U. S. A.*, 1995, **92**, 372–76.
30 J. Heberle, Proton transfer reactions across bacteriorhodopsin and along the membrane, *Biochim. Biophys. Acta*, 2000, **1458**, 135–47.
31 F. Garczarek, L. S. Brown, J. K. Lanyi and K. Gerwert, Proton binding within a membrane protein by a protonated water cluster, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 3633–38.
32 C. A. Wraight, Electron acceptors of bacterial photosynthetic reaction centers. II. Proton, binding coupled to secondary electron transfer in the quinone acceptor complex, *Biochim. Biophys. Acta*, 1979, **548**, 309–27.
33 P. H. McPherson, M. Y. Okamura and G. Feher, Light induced proton uptake by photosynthetic reaction centers from *Rhodobacter spheroides* R-26. I. Protonation, of the one-electron-states, *Biochim. Biophys. Acta*, 1988, **934**, 348–68.
34 O. A. Gopta, D. A. Cherepanov, W. Junge and A. Y. Mulkidjanian, Proton transfer from the bulk to the bound ubiquinone Q_B of the reaction center in chromatophores of *Rhodobacter sphaeroides*: retarded conveyance by neutral water, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 13159–64.
35 M. L. Paddock, G. Feher and M. Y. Okamura, Proton transfer pathways and mechanism in bacterial reaction centers, *FEBS Lett.*, 2003, **555**, 45–50.
36 A. Y. Mulkidjanian, D. A. Cherepanov and M. A. Kozlova, Ubiquinone reduction in the photosynthetic reaction center of *Rhodobacter sphaeroides*: Interplay between electron transfer, proton binding and the flips of quinone ring, *Biochem. Soc. Trans.*, 2005, **33**, 845–50.
37 C. A. Wraight, Intraprotein Proton Transfer: Concepts and Realities from the Bacterial Photosynthetic Reaction Center, in *Biophysical and Structural Aspects of Bioenergetics*, ed. M. Wikstrom, Royal Society of Chemistry, Cambridge, 2005, pp. 273–312.
38 W. Ausländer and W. Junge, Electric generator in the photosynthesis of green plants. II. Kinetic, correlation between protolytic reactions and redox reactions, *Biochim. Biophys. Acta*, 1974, **357**, 285–98.
39 J. Lavergne and W. Junge, Proton release during the redox cycle of the water oxidase, *Photosynthesis Res.*, 1993, **38**, 279–96.
40 M. Haumann and W. Junge, Extent and rate of proton release by photosynthetic water oxidation in thylakoids: electrostaticrelaxation *versus* chemical production, *Biochemistry*, 1994, **33**, 864–72.

41 M. Haumann and W. Junge, The rates of proton uptake and electron transfer at the reducing side of photosystem II in thylakoids, *FEBS Lett.*, 1994, **347**, 45–50.
42 M. Haumann, A. Mulkidjanian and W. Junge, Electrogenicity of electron and proton transfer at the oxidizing side of photosystem II, *Biochemistry*, 1997, **36**, 9304–15.
43 K. M. Petty and P. L. Dutton, Ubiquinone-cytochrome *b* electron and proton transfer: a functional pK on cytochrome b50 in *Rhodopseudomonas sphaeroides* membranes, *Arch. Biochem. Biophys.*, 1976, **172**, 346–53.
44 S. Saphon and P. Graber, External proton uptake, internal proton release and internal pH changes in chromatophores from *Rps. sphaeroides* following single turnover flashes, *Z. Naturforsch., Teil C*, 1978, **33C**, 715–22.
45 A. Y. Mulkidjanian and W. Junge, Calibration and time resolution of lumenal pH-transients in chromatophores of *Rhodobacter capsulatus* following a single turnover flash of light: proton release by the cytochrome bc_1-complex is strongly electrogenic, *FEBS Lett.*, 1994, **353**, 189–93.
46 S. S. Klishin and A. Y. Mulkidjanian, Proton transfer paths at the quinol oxidizing site of the *Rb. capsulatus* cytochrome *bc* complex, in *Photosynthesis: Fundamental aspects to global perspectives,*, ed. A. van der Est and D. Bruce, Allen Press Inc., Lawrence/Montreal, 2005, vol. 1, pp. 260–262.
47 A. Jasaitis, V. B. Borisov, N. P. Belevich, J. E. Morgan, A. A. Konstantinov and M. I. Verkhovsky, Electrogenic reactions of cytochrome *bd*, *Biochemistry*, 2000, **39**, 13800–09.
48 M. Wikstrom and M. I. Verkhovsky, Proton translocation by cytochrome c oxidase in different phases of the catalytic cycle, *Biochim. Biophys. Acta*, 2002, **1555**, 128–32.
49 P. Brzezinski and G. Larsson, Redox-driven proton pumping by heme-copper oxidases, *Biochim. Biophys. Acta*, 2003, **1605**, 1–13.
50 R. M. Nyquist, D. Heitbrink, C. Bolwien, R. B. Gennis and J. Heberle, Direct observation of protonation reactions during the catalytic cycle of cytochrome c oxidase, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 8715–20.
51 D. Bloch, I. Belevich, A. Jasaitis, C. Ribacka, A. Puustinen, M. I. Verkhovsky and M. Wikstrom, The catalytic cycle of cytochrome c oxidase is not the sum of its two halves, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 529–33.
52 D. Zaslavsky, R. C. Sadoski, S. Rajagukguk, L. Geren, F. Millett, B. Durham and R. B. Gennis, Direct measurement of proton release by cytochrome c oxidase in solution during the F → O transition, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 10544–47.
53 I. Belevich, V. B. Borisov, J. Zhang, K. Yang, A. A. Konstantinov, R. B. Gennis and M. I. Verkhovsky, Time-resolved electrometric and optical studies on cytochrome *bd* suggest a mechanism of electron-proton coupling in the di-heme active site, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 3657–62.
54 K. Faxen, G. Gilderson, P. Adelroth and P. Brzezinski, A mechanistic principle for proton pumping by cytochrome c oxidase, *Nature*, 2005, **437**, 286–89.
55 J. H. Hendriks, A. Jasaitis, M. Saraste and M. I. Verkhovsky, Proton and electron pathways in the bacterial nitric oxide reductase, *Biochemistry*, 2002, **41**, 2331–40.
56 S. Saphon, J. B. Jackson and H. T. Witt, Electrical potential changes, H^+ translocation and phosphorylation induced by short flash excitation in *Rhodopseudomonas sphaeroides* chromatophores, *Biochim. Biophys. Acta*, 1975, **408**, 67–82.
57 W. Junge, Complete tracking of transient proton flow through active chloroplast ATP synthase, *Proc. Natl. Acad. Sci. U. S. A.*, 1987, **84**, 7084–88.
58 B. A. Feniouk, D. A. Cherepanov, W. Junge and A. Y. Mulkidjanian, Coupling of proton flow to ATP synthesis in *Rhodobacter capsulatus*: F_OF_1-ATPsynthase is absent from about half of chromatophores, *Biochim. Biophys. Acta*, 2001, **1506**, 189–203.
59 B. A. Feniouk, M. A. Kozlova, D. A. Knorre, D. A. Cherepanov, A. Y. Mulkidjanian and W. Junge, The proton driven rotor of ATP synthase: Ohmic conductance (10 fS), and absence of voltage gating, *Biophys. J.*, 2004, **86**, 4094–109.
60 A. Y. Mulkidjanian, D. A. Cherepanov, J. Heberle and W. Junge, Proton transfer dynamics at membrane/water interface and mechanism of biological energy conversion, *Biochemistry (Moscow)*, 2005, **70**, 251–56.
61 A. Y. Mulkidjanian, J. Heberle and D. A. Cherepanov, Protons @ interfaces: Implications for biological energy conversion, *Biochim. Biophys. Acta*, 2006, DOI: 10.1016/j.bbabio.2006.02.015.
62 H. Arata, I. Takenaka and M. Nishimura, Flash-induced proton release in *Rhodopseudomonas sphaeroides* spaeroplasts, *J. Biochem.*, 1987, **101**, 261–65.
63 M. R. Jones and J. B. Jackson, Proton release by the quinol oxidase site of the cytochrome b/c_1 complex following single turnover flash exitation of intact cells of *Rhodobacter capsulatus*, *Biochim. Biophys. Acta*, 1989, **975**, 34–43.
64 M. R. Jones and J. B. Jackson, Proton efflux from right-side-out membrane vesicles of *Rhodobacter sphaeroides* after short flashes, *Biochim. Biophys. Acta*, 1990, **1019**, 51–58.
65 J. Heberle Zeitauflösende, *Untersuchung der Protonentranslokationsschritte von Bakteriorhodopsin mittels chemisch-gekoppelter pH-Indikatoren*, Freien Universität, Berlin, 1991.
66 L. A. Drachev, A. D. Kaulen and A. Y. Komrakov, Interrelations of M-intermediates in bacteriorhodopsin photocycle, *FEBS Lett.*, 1992, **313**, 248–50.
67 P. Scherrer, U. Alexiev, T. Marti, H. G. Khorana and M. P. Heyn, Covalently bound pH-indicator dyes at selected extracellular or cytoplasmic sites in bacteriorhodopsin. 1. Proton migration along the surface of bacteriorhodopsin micelles and its delayed transfer from surface to bulk, *Biochemistry*, 1994, **33**, 13684–92.
68 P. Maroti and C. A. Wraight, Kinetics of H^+ ion binding by the $P^+Q_A^-$ state of bacterial photosynthetic reaction centers: rate limitation within the protein, *Biophys. J.*, 1997, **73**, 367–81.
69 Y. N. Antonenko and P. Pohl, Coupling of proton source and sink *via* H^+-migration along the membrane surface as revealed by double patch-clamp experiments, *FEBS Lett.*, 1998, **429**, 197–200.
70 D. Porschke, Reaction coupling, acceptor p*K*, and diffusion control in light induced proton release of bacteriorhodopsin, *J. Phys. Chem. B*, 2002, **106**, 10233–41.
71 Y. Georgievskii, E. S. Medvedev and A. A. Stuchebrukhov, Proton transport *via* the membrane surface, *Biophys. J.*, 2002, **82**, 2833–46.
72 D. A. Cherepanov, W. Junge and A. Y. Mulkidjanian, Proton transfer dynamics at the membrane/water interface: Dependence on the fixed and mobile pH buffers, on the size and form of membrane particles, and on the interfacial potential barrier, *Biophys. J.*, 2004, **86**, 665–80.
73 B. A. Feniouk, D. A. Cherepanov, W. Junge and A. Y. Mulkidjanian, Anisotropy of proton fluxes at the membrane/water interface and its role in ATP synthesis by Rhodobacter capsulatus chromatophores, in *EBEC Short Reports*, Elsevier, Arcachon, 2002, vol. 12, p. 40.
74 D. A. Cherepanov, B. A. Feniouk, W. Junge and A. Y. Mulkidjanian, Low dielectric permittivity of water at the membrane interface: Effect on the energy coupling mechanism in biological membranes, *Biophys. J.*, 2003, **85**, 1307–16.
75 D. A. Cherepanov, Force oscillations and dielectric overscreening of interfacial water, *Phys. Rev. Lett.*, 2004, **93**, 266104.
76 W. Junge, Flash kinetic spectrophotometry in the study of plant pigments, in *Chemistry and Biochemistry of Plant Pigments*, ed. T. W. Goodwin, Academic Press, London, 1976, pp. 233–333.
77 C. A. Wraight, R. J. Cogdell and B. Chance, Ion transport and electrochemical gradients in photosynthetic bacteria, in *The Photosynthetic Bacteria*, ed. R. K. Clayton and W. R. Sistrom, Academic Press, New York, 1978, pp. 471–511.
78 L. A. Drachev, A. D. Kaulen, A. Y. Semenov, I. I. Severina and V. P. Skulachev, Lipid-impregnated filters as a tool for studying the electric current-generating proteins, *Anal. Biochem.*, 1979, **96**, 250–62.
79 B. Chance, J. A. McCray and J. Bunkenburg, Fast spectrophotometric measurement of H^+ changes in *Chromatium* chromatophores activated by a liquid dye laser, *Nature*, 1970, **225**, 705–08.
80 B. Chance, A. R. Crofts, M. Nishimura and B. Price, Fast membrane H^+ binding in the light-activated state of *Chromatium* chromatophores, *Eur. J. Biochem.*, 1970, **13**, 364–74.
81 A. R. Crofts, R. J. Codgell and J. B. Jackson, The mechanism of H^+ uptake in *Rhodopseudomonas sphaeroides*, in *Energy Transduction in Respiration and Photosynthesis*, ed. E. Quagliariello, S. Papa and C. S. Rossi, Adriatica Editrici, Bari, 1971, pp. 883–901.
82 R. J. Codgell, J. B. Jackson and A. R. Crofts, The effect of redox potential on the coupling between rapid hydrogen-ion binding and electron transport in chromatophores from *Rhodobacter sphaeroides*, *Bioenergetics*, 1972, **4**, 413–29.
83 K. M. Petty and P. L. Dutton, Properties of the Flash-Induced Proton Binding Encountered in Membranes of *Rhodopseudomonas*

sphaeroides: A Functional p*K* on the Ubisemiquinone?, *Arch. Biochem. Biophys.*, 1976, **172**, 335–45.
84 A. K. Dioumaev, H. T. Richter, L. S. Brown, M. Tanio, S. Tuzi, H. Saito, Y. Kimura, R. Needleman and J. K. Lanyi, Existence of a proton transfer chain in bacteriorhodopsin: participation of Glu-194 in the release of protons to the extracellular surface, *Biochemistry*, 1998, **37**, 2496–506.
85 W. Junge and A. Polle, Theory of proton flow along appressed thylakoid membranes under both non-stationary and stationary conditions, *Biochim. Biophys. Acta*, 1986, **848**, 265–73.
86 W. Junge and S. McLaughlin, The role of fixed and mobile buffers in the kinetics of proton movement, *Biochim. Biophys. Acta*, 1987, **890**, 1–5.
87 J. Heberle and N. A. Dencher, The surface of the purple membrane: a transient pool for protons, in *Structures and Functions of Retinal Proteins*, ed. J. L. Rigaud, John Libbey Eurotext Ltd., London, 1992, pp. 221–224.
88 E. Nachliel and M. Gutman, Quantitative evaluation of the dynamics of proton transfer from photoactivated bacteriorhodopsin to the bulk, *FEBS Lett.*, 1996, **393**, 221–25.
89 J. Riesle, D. Oesterhelt, N. A. Dencher and J. Heberle, D38 is an essential part of the proton translocation pathway in bacteriorhodopsin, *Biochemistry*, 1996, **35**, 6635–43.
90 B. Schatzler, N. A. Dencher, J. Tittor, D. Oesterhelt, S. Yaniv-Checover, E. Nachliel and M. Gutman, Subsecond proton-hole propagation in bacteriorhodopsin, *Biophys. J.*, 2003, **84**, 671–86.
91 S. Checover, Y. Marantz, E. Nachliel, M. Gutman, M. Pfeiffer, J. Tittor, D. Oesterhelt and N. A. Dencher, Dynamics of the proton transfer reaction on the cytoplasmic surface of bacteriorhodopsin, *Biochemistry*, 2001, **40**, 4281–92.
92 P. Adelroth and P. Brzezinski, Surface-mediated proton-transfer reactions in membrane-bound proteins, *Biochim. Biophys. Acta*, 2004, **1655**, 102–15.
93 S. Grzesiek and N. A. Dencher, Dependency of delta pH-relaxation across vesicular membranes on the buffering power of bulk solutions and lipids, *Biophys. J.*, 1986, **50**, 265–76.
94 J. K. Beattie, General Discussion, *Faraday Discuss.*, 2005, **129**, 89–109.
95 M. Eigen, Protonenübertragung, Säure-Base-Katalyse and enzymatische Hydrolyse.Teil I: Elementarvorgänge, *Angew. Chem.*, 1963, **75**, 489–588.
96 R. P. Bell, *The Proton in Chemistry*, Chapman & Hall, London, 1973.
97 N. Agmon, The Grotthuß Mechanism, *Chem. Phys. Lett.*, 1995, **244**, 456–62.
98 P. Raiteri, A. Laio and M. Parrinello, Correlations among hydrogen bonds in liquid water, *Phys. Rev. Lett.*, 2004, **93**, 087801.
99 J. O. Bockris and A. K. N. Reddy, *Modern Electrochemistry*, Plenum Press, New York, 1977.
100 P. I. Lelkes and I. R. Miller, Perturbations of membrane structure by optical probes: I. Location, and structural sensitivity of merocyanine 540 bound to phospholipid membranes, *J. Membr. Biol*, 1980, **52**, 1–15.
101 G. Cevc, A. Watts and D. Marsh, Titration of the phase transition of phosphatidylserine bilayer membranes. Effects of pH, surface electrostatics, ion binding, and head-group hydration, *Biochemistry*, 1981, **20**, 4955–65.
102 V. B. P. Leite, A. Cavalli and O. N. Oliveira Jr., Hydrogen-bond control of structure and conductivity of Langmuir films, *Phys. Rev. E*, 1998, **57**, 6835–39.
103 O. N. Oliveira Jr., A. Riul and V. B. P. Leite, Water at interfaces and its influence on the electrical properties of adsorbed films, *Braz. J. Phys.*, 2004, **34**, 73–83.
104 C. J. Slevin and P. R. Unwin, Lateral proton diffusion rates along stearic acid monolayers, *J. Am. Chem. Soc.*, 2000, **122**, 2597–602.
105 J. Zhang and P. R. Unwin, Proton diffusion at phospholipid assemblies, *J. Am. Chem. Soc.*, 2002, **124**, 2379–83.
106 H. I. Petrache, S. Tristram-Nagle, K. Gawrisch, D. Harries, V. A. Parsegian and J. F. Nagle, Structure and fluctuations of charged phosphatidylserine bilayers in the absence of salt, *Biophys. J.*, 2004, **86**, 1574–86.
107 J. Fitter, R. E. Lechner and N. A. Dencher, Interactions of hydration water and biological membranes studied by neutron scattering, *J. Phys. Chem. B*, 1999, **103**, 8036–50.
108 S. Braun-Sand, M. Strajbl and A. Warshel, Studies of proton translocations in biological systems: simulating proton transport in carbonic anhydrase by EVB-based models, *Biophys. J.*, 2004, **87**, 2221–39.
109 T. Ishino, H. Hieda, K. Tanaka and N. Gemma, Measurements of electrostatic double-layer forces due to charged functional groups on Langmuir–Blogett films with an atomic force microscope, *Jpn. J. Appl. Phys.*, 1994, **33**, 4718–22.
110 P. A. Bopp, A. A. Kornyshev and G. Sutmann, Static nonlocal dielectric function of liquid water, *Phys. Rev. Lett.*, 1996, **76**, 1280–83.
111 P. A. Bopp, A. A. Kornyshev and G. Sutmann, Frequency and wavevector dependent dielectric function of water: Collective modes and relaxation spectra, *J. Chem. Phys.*, 1998, **109**, 1939–59.
112 G. Cevc, Molecular-force theory of solvation of the polar solutes- the mean field solvation model, its implications and examples from lipid water mixtures, *Chem. Scr.*, 1985, **25**, 99.
113 M. L. Belaya, M. V. Feigelman and V. G. Levadny, Hydration forces as a result of nonlocal water polarizability, *Chem. Phys. Lett.*, 1986, **126**, 361–64.
114 S. Leikin and A. A. Kornyshev, Theory of hydration forces. Nonlocal electrostatic interaction of neutral surfaces, *J. Chem. Phys.*, 1990, **92**, 6890–98.
115 A. A. Kornyshev, Nonlocal electrostatics of solvation, in *The Chemical Physics of Solvation*, ed. R. R. Dogonadze, E. Kalman, A. A. Kornyshev and J. Ulstrup, Elsevier, Amsterdam, 1985, pp. 77–118.
116 P. G. Dzhavakhidze, A. A. Kornyshev and V. K. Levadny, The structure of the interface in the solvent-mediated interaction of dipolar surfaces, *Nuovo Cimento Soc. Ital. Fis., D*, 1988, **10**, 627–53.
117 I. P. Omelyan, Longitudinal wavevector- and frequency-dependent dielectric constant of the TIP4P water model, *Mol. Phys.*, 1998, **93**, 123–35.
118 O. V. Dolgov, D. A. Kirzhnits and E. G. Maksimov, On an admissible sign of the static dielectric function of matter, *Rev. Mod. Phys.*, 1981, **53**, 81–93.
119 V. L. Ginzburg, Nobel Lecture: On conductivity and superfluidity, *Rev. Mod. Phys.*, 2004, **76**.
120 A. A. Kornyshev, S. Leikin and G. Sutmann, ''Overscreening'' in a polar liquid as a result of coupling between polarization and density fluctuations, *Electrochim. Acta*, 1997, **42**, 849–65.
121 U. Raviv, P. Laurat and J. Klein, Fluidity of water confined to subnanometre films, *Nature*, 2001, **413**, 51–54.
122 Y. X. Zhu and S. Granick, Viscosity of interfacial water, *Phys. Rev. Lett.*, 2001, **8709**, 096104.
123 U. Raviv and J. Klein, Fluidity of bound hydration layers, *Science*, 2002, **297**, 1540–43.
124 J. N. Israelachvili, P. M. Mcguiggan and A. M. Homola, Dynamic Properties of Molecularly Thin Liquid-Films, *Science*, 1988, **240**, 189–91.
125 S. Granick, Motions and Relaxations of Confined Liquids, *Science*, 1991, **253**, 1374–79.
126 R. E. Lechner and N. A. Dencher, Two-dimensional proton diffusion on purple membrane, *Solid State Ionics*, 1994, **70/71**, 296–304.
127 J. N. Israelachvili and R. M. Pashley, Molecular layering of water at surfaces and origin of repulsive hydration forces, *Nature*, 1983, **306**, 249–50.
128 J. P. Cleveland, T. E. Schaffer and P. K. Hansma, Probing oscillatory hydration potentials using thermal-mechanical noise in an atomic force microscope, *Phys. Rev. B*, 1995, **52**, R8692–R8695.
129 J. N. Israelachvili and H. Wennerstrom, Role of hydration and water structure in biological and colloidal interactions, *Nature*, 1996, **379**, 219–25.
130 G. E. Jr, Brown, How minerals react with water, *Science*, 2001, **294**, 67–70.
131 A. J. Archer and R. Evans, Binary Gaussian core model: Fluid-fluid phase separation and interfacial properties, *Phys. Rev. E*, 2001, **6404**.
132 R. M. Pashley and J. N. Israelachvili, Molecular layering of water in thin-films between mica surfaces and its relation to hydration forces, *J. Colloid Interface Sci.*, 1984, **101**, 511–23.
133 E. A. Vogler, Structure and reactivity of water at biomaterial surfaces, *Adv. Colloid Interface Sci.*, 1998, **74**, 69–117.
134 K. D. Garlid, The state of water in biological systems, *Int. Rev. Cytol.*, 2000, **192**, 281–302.
135 R. C. Ford, S. V. Ruffle, A. J. Ramirez-Cuesta, I. Michalarias, I. Beta, A. Miller and J. Li, Inelastic incoherent neutron scattering measurements of intact cells and tissues and detection of interfacial water, *J. Am. Chem. Soc.*, 2004, **126**, 4682–88.

136 P. Mitchell, Coupling of photophosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism, *Nature*, 1961, **191**, 144–48.
137 P. Mitchell, Chemiosmotic coupling in oxidative and photosynthetic phosphorylation, *Physiol. Rev.*, 1966, **41**, 445–502.
138 W. A. Cramer and D. B. Knaff, *Energy transduction in biological membranes: a textbook of bioenergetics*, Springer, New York, 1991.
139 D. B. Kell, On the functional proton current pathway of electron transport phosphorylation. An electrodic view, *Biochim. Biophys. Acta*, 1979, **549**, 55–99.
140 S. J. Ferguson, Fully delocalised chemiosmotic or localised proton flow pathways in energy coupling? A scrutiny of experimental evidence, *Biochim. Biophys. Acta*, 1985, **811**, 47–95.
141 H. V. Westerhoff, B. A. Melandri, G. Venturoli, G. F. Azzone and D. B. Kell, A minimal hypothesis for membrane-linked free-energy transduction. The role of independent, small coupling units, *Biochim. Biophys. Acta*, 1984, **768**, 257–92.
142 D. B. Kell, Localized protonic coupling: overview and critical evaluation of techniques., *Methods Enzymol.*, 1986, **127**, 538–57.
143 T. A. Krulwich, M. Ito, R. Gilmour, D. B. Hicks and A. A. Guffanti, Energetics of alkaliphilic Bacillus species: physiology and molecules, *Adv. Microb. Physiol.*, 1998, **40**, 401–38.
144 E. R. Decker and D. G. Levitt, Use of weak acids to determine the bulk diffusion limitationof H^+ ion conductance through the gramicidin channel, *Biophys. J.*, 1988, **53**, 25–32.
145 S. Cukierman, Proton mobilities in water and in different stereoisomers of covalently linked gramicidin A channels, *Biophys. J.*, 2000, **78**, 1825–34.
146 O. S. Andersen, Ion movement through gramicidin A channels. Studies on the diffusion-controlled association step, *Biophys. J.*, 1983, **41**, 147–65.
147 F. A. Schabert and A. Engel, Reproducible acquisition of *Escherichia coli* porin surface topographs by atomic force microscopy, *Biophys. J.*, 1994, **67**, 2394–403.
148 R. M. Sassaki, R. A. Douglas, M. U. Kleinke and O. Teschke, Structure imaging by atomic force microscopy and transmission electron microscopy of different light emitting species of porous silicon, *J. Vac. Sci. Technol., B*, 1996, **14**, 2432–37.
149 D. J. Muller and A. Engel, The height of biomolecules measured with the atomic force microscope depends on electrostatic interactions, *Biophys. J.*, 1997, **73**, 1633–44.
150 M. K. Gilson, A. Rashin, R. Fine and B. Honig, On the calculation of electrostatic interactions in proteins, *J. Mol. Biol.*, 1985, **184**, 503–16.
151 A. Finkelstein and O. Ptitsyn, *Protein Physics: A Course of Lectures*, Academic Press, London & San Diego, 2002.
152 J. Antosiewicz, J. A. McCammon and M. K. Gilson, The determinants of pK_as in proteins, *Biochemistry*, 1996, **35**, 7819–33.
153 D. A. Cherepanov, S. I. Bibikov, M. V. Bibikova, D. A. Bloch, L. A. Drachev, O. A. Gopta, D. Oesterhelt, A. Y. Semenov and A. Y. Mulkidjanian, Reduction and protonation of the secondary quinone acceptor of *Rhodobacter sphaeroides* photosynthetic reaction center: kinetic model based on a comparison of wild-type chromatophores with mutants carrying Arg → Ile substitution at sites 207 and 217 in the L-subunit, *Biochim. Biophys. Acta*, 2000, **1459**, 10–34.
154 T. Simonson, J. Carlsson and D. A. Case, Proton binding to proteins: pK_a calculations with explicit and implicit solvent models, *J. Am. Chem. Soc.*, 2004, **126**, 4167–80.
155 B. Kuhn, P. A. Kollman and M. Stahl, Prediction of pK_a shifts in proteins using a combination of molecular mechanical and continuum solvent calculations, *J. Comput. Chem.*, 2004, **25**, 1865–72.
156 R. B. Silver, Imaging structured space-time patterns of Ca^{2+} signals: essential information for decisions in cell division, *FASEB J.*, 1999, **13**(Suppl. 2), S209–S215.
157 R. J. Williams, The structures of organelles and reticula: localised bioenergetics and metabolism, *ChemBioChem*, 2001, **2**, 637–41.
158 S. V. Meethal, K. T. Potter, D. Redon, D. M. Heisey and R. A. Haworth, Ca transients from Ca channel activity in rat cardiac myocytes reveal dynamics of dyad cleft and troponin C Ca binding, *Am. J. Physiol.: Cell Physiol.*, 2004, **286**, C302–C316.
159 M. Vendelin, M. Lemba and V. A. Saks, Analysis of functional coupling: mitochondrial creatine kinase and adenine nucleotide translocase, *Biophys. J.*, 2004, **87**, 696–713.
160 M. Vendelin, M. Eimre, E. Seppet, N. Peet, T. Andrienko, M. Lemba, J. Engelbrecht, E. K. Seppet and V. A. Saks, Intracellular diffusion of adenosine phosphates is locally restricted in cardiac muscle, *Mol. Cell. Biochem.*, 2004, **256–257**, 229–41.
161 E. Pebay-Peyroula, C. Dahout-Gonzalez, R. Kahn, V. Trezeguet, G. J. Lauquin and G. Brandolin, Structure of mitochondrial ADP/ATP carrier in complex with carboxyatractyloside, *Nature*, 2003, **426**, 39–44.
162 C. R. Lancaster, A. Kroger, M. Auer and H. Michel, Structure of fumarate reductase from Wolinella succinogenes at 2.2 Å resolution, *Nature*, 1999, **402**, 377–85.
163 T. M. Iverson, C. Luna-Chavez, G. Cecchini and D. C. Rees, Structureof the Escherichia coli fumarate reductase respiratory complex, *Science*, 1999, **284**, 1961–66.
164 V. Yankovskaya, R. Horsefield, S. Tornroth, C. Luna-Chavez, H. Miyoshi, C. Leger, B. Byrne, G. Cecchini and S. Iwata, Architecture of succinate dehydrogenase and reactive oxygen species generation, *Science*, 2003, **299**, 700–04.
165 M. G. Bertero, R. A. Rothery, M. Palak, C. Hou, D. Lim, F. Blasco, J. H. Weiner and N. C. Strynadka, Insights into the respiratory electron transfer pathway from the structure of nitrate reductase A, *Nat. Struct. Biol*, .2003, **10**, 681–87.
166 C. R. Lancaster and J. Simon, Succinate:quinone oxidoreductases from epsilon-proteobacteria, *Biochim. Biophys. Acta*, 2002, **1553**, 84–101.
167 D. Stock, C. Gibbons, I. Arechaga, A. G. Leslie and J. E. Walker, The rotary mechanism of ATP synthase, *Curr. Opin. Struct. Biol*, .2000, **10**, 672–79.
168 M. Yoshida, E. Muneyuki and T. Hisabori, ATP synthase- a marvellous rotary engine of the cell, *Nat. Rev. Mol. Cell Biol.*, 2001, **2**, 669–77.
169 D. A. Cherepanov and W. Junge, Viscoelastic dynamics of actin filaments coupled to rotary F-ATPase: curvature as an indicator of the torque, *Biophys. J.*, 2001, **81**, 1234–44.
170 H. Stahlberg, D. J. Muller, K. Suda, D. Fotiadis, A. Engel, T. Meier, U. Matthey and P. Dimroth, Bacterial Na^+-ATP synthase has an undecameric rotor, *EMBO Rep.*, 2001, **2**, 229–33.
171 C. Toyoshima, M. Nakasako, H. Nomura and H. Ogawa, Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 Å resolution, *Nature*, 2000, **405**, 647–55.
172 N. P. Cotton, S. A. White, S. J. Peake, S. McSweeney and J. B. Jackson, The crystal structure of an asymmetric complex of the two nucleotide binding components of proton-translocating transhydrogenase, *Structure*, 2001, **9**, 165–76.
173 K. P. Locher, A. T. Lee and D. C. Rees, The *E. coli* BtuCD, structure: a framework for ABC transporter architecture and mechanism, *Science*, 2002, **296**, 1091–98.
174 R. J. Ellis, Macromolecular crowding: an important but neglected aspect of the intracellular environment, *Curr. Opin. Struct. Biol.*, 2001, **11**, 114–19.
175 A. S. Verkman, Solute and macromolecule diffusion in cellular aqueous compartments, *Trends Biochem. Sci.*, 2005, **27**, 27–33.
176 M. H. Stowell, T. M. McPhillips, D. C. Rees, S. M. Soltis, E. Abresch and G. Feher, Light-induced structural changes in photosynthetic reaction center: implications for mechanism of electron-proton transfer, *Science*, 1997, **276**, 812–16.
177 L. Essen, R. Siegert, W. D. Lehmann and D. Oesterhelt, Lipid patches in membrane protein oligomers: crystal structure of the bacteriorhodopsin-lipid complex, *Proc. Natl. Acad. Sci. U. S. A.*, 1998, **95**, 11673–78.
178 W. Humphrey, A. Dalke and K. Schulten, VMD: Visual molecular dynamics, *J. Mol. Graphics*, 1996, **14**, 33–38.
179 R. I. Menz, J. E. Walker and A. G. Leslie, Structure of bovine mitochondrial F_1-ATPase with nucleotide bound to all three catalytic sites: implications for the mechanism of rotary catalysis, *Cell*, 2001, **106**, 331–41.
180 T. Meier, P. Polzer, K. Diederichs, W. Welte and P. Dimroth, Structure of the rotor ring of F-Type Na^+-ATPase from *Ilyobacter tartaricus*, *Science*, 2005, **308**, 659–62.